VIEWPOINT

Clinical proteomics: A need to define the field and to begin to set adequate standards

Harald Mischak[1], Rolf Apweiler[2], Rosamonde E. Banks[3], Mark Conaway[4], Joshua Coon[5], Anna Dominiczak[6], Jochen H. H. Ehrich[7], Danilo Fliser[8], Mark Girolami[9], Henning Hermjakob[2], Denis Hochstrasser[10, 11], Joachim Jankowski[12], Bruce A. Julian[13], Walter Kolch[14], Ziad A. Massy[15], Christian Neususess[16], Jan Novak[17], Karlheinz Peter[18], Kasper Rossing[19], Joost Schanstra[20], O. John Semmes[21], Dan Theodorescu[22], Visith Thongboonkerd[23], Eva M. Weissinger[24], Jennifer E. Van Eyk[25] and Tadashi Yamamoto[26]

[1] Mosaiques Diagnostics, Hannover, Germany*

The aim of this manuscript is to initiate a constructive discussion about the definition of clinical proteomics, study requirements, pitfalls and (potential) use. Furthermore, we hope to stimulate proposals for the optimal use of future opportunities and seek unification of the approaches in clinical proteomic studies. We have outlined our collective views about the basic principles that should be considered in clinical proteomic studies, including sample selection, choice of technology and appropriate quality control, and the need for collaborative interdisciplinary efforts involving clinicians and scientists. Furthermore, we propose guidelines for the critical aspects that should be included in published reports. Our hope is that, as a result of stimulating discussion, a consensus will be reached amongst the scientific community leading to guidelines for the studies, similar to those already published for mass spectrometric sequencing data. We contend that clinical proteomics is not just a collection of studies dealing with analysis of clinical samples. Rather, the essence of clinical proteomics should be to address clinically relevant questions and to improve the state-of-the-art, both in diagnosis and in therapy of diseases.

Received: October 11, 2006
Revised: December 3, 2006
Accepted: December 5, 2006

Keywords:
Biomarker / Clinical diagnostics / Protein marker discovery / Protein profiling / Proteome analysis

Introduction

In the post-genomics era, the literature has been flooded with manuscripts dealing with clinical proteomics. Unfortunately, many of these reports are of questionable value and cannot be interpreted adequately or reproduced for a number of reasons. These include the lack of consensus practices regarding appropriate study design(s) and of scientific rigour, particularly regarding the study/cohort design, use of sample sizes of sufficient statistical power, sample processing and other pre-analytical aspects, associated clinical data, appropriate quality control, statistical evaluation and independent validation and, in some cases, all of the above. These shortcomings appear to have frequently resulted from an absence of interdisciplinary interactions that are crucial for obtaining value from "clinical proteomics". In an effort to improve this situation and avoid undesirable developments, we have attempted to outline the principal defining parameters for a clinical proteomics study and highlight potential problems and pitfalls. In the interest of clarity and to stay focused, we chose to abstain

Correspondence: Dr. Harald Mischak, Mosaiques Diagnostics & Therapeutics AG, Mellendorfer Str. 7–9, 30625 Hannover, Germany
E-mail: mischak@mosaiques-diagnostics.com
Fax: +49-511-554744-31

Abbreviations: **GCP**, good clinical practice; **GCLP**, good clinical laboratory practice

* For other affiliations please see Addendum

from covering several important technical issues, such as the limitations and advantages of the different proteomics platforms or validation of biomarkers in large-scale clinical studies, which have been discussed in several review articles that also carry more detailed consideration of several of the aspects we describe below [1, 2].

In addition to a patient history and physical examination, clinical diagnosis traditionally depends on ascertainment of biophysical and biochemical markers such as blood pressure or levels of cholesterol, hormones and metabolites, or images of physical traits. The issue of discovery and validation of biomarkers is the subject of many studies and an illustration of this process can be gained from reviewing prostate-specific antigen (PSA) for example [3]. While our current clinical biomarker arsenal has convincingly demonstrated its usefulness, the process of biomarker discovery has been mainly serendipitous rather than a result of large-scale systematic investigation. Although such markers may have been in widespread use for many years, *e.g.* PSA, there still remain unresolved issues with regard to its optimal clinical use [3]. Clearly, we need more and better markers to complement or replace the existing ones in many areas. Genomics and proteomics, at least in principle, hold promise for an unbiased, systematic discovery route, and thus have rapidly become popular. Genetic phenotyping focuses on hereditary predisposition, but other biomarker sources are needed to detect when and to what extent the risks have become manifest, and how overt disease will respond to therapy, or progress. Gene expression analysis allows the determination of transcript levels for many thousands of genes, but this does not necessarily equate to production of functional protein and provides no information about post-translational events that may often change in disease. This problem may be directly addressed by proteomics, which can potentially capture dynamic changes in protein expression, integrating both genetic and epigenetic influences. As the proteome is far more extensive than the genome, it offers a richer source of potential biomarkers. However, this conceptual advantage of proteomics also present enormous challenges, as the levels of complexity and dynamic ranges in body fluids are difficult to measure and analyse, and often require the development of new technological and bioinformatics solutions. Even so, we are currently in a position to take a critical account of the results and lessons learned in order to rethink our expectations of clinical proteomics and perhaps redefine future strategies.

Will proteome analysis realistically enable the discovery and definition of new biomarkers for early diagnosis of disease, improvement in disease staging and grading or prediction of outcome? Just a few years ago this was the central question. As a consequence, "clinical" samples (*e.g.* tissue, cells, body fluids) from patients and controls were enthusiastically analysed using a range of different proteomic-based technological approaches. Even with rather crude methods, the data showed differences between patients and controls, and consequently potential biomarkers for disease, in a large number of studies. However, not only disease-specific biomarkers, but also "biomarkers" indicating inter-individual biological differences (*e.g.* between control A and control B) could be observed (Mischak *et al.*, unpublished observation). Such "biomarkers" reflected not only biological, but also technical variability (*e.g.* pre-analytical and analytical influences) and would likely be confounders for disease diagnostics. The poor quality of many of the early studies was damaging to the field, and led to a questioning of the viability of the initially defined concept of "disease-specific biomarkers". However, the upside is that this work highlighted the requirement for precise sample handling, minimal technical variation, stringent statistics and robust study design. These features are essential for comparability of different studies and of utmost importance in clinical proteomics. The various stages and requirements of the development pipeline for biomarkers or "*in vitro* diagnostics" generally have been recently reviewed in detail [1, 2, 4, 5].

Clinical proteomics should be defined as the application of proteomic analysis with the aim of solving a specific clinical problem within the context of a clinical study

A clinical proteomic study should begin with a well-framed clinical question or problem, followed by selection of the appropriate study populations, samples to be analysed, and technology to analyse the samples. The goals of such studies may include earlier or more accurate diagnosis, improvement of therapeutic strategies, and better evaluation of prognosis and/or prevention of the disease. Although currently the main focus is diagnostics and biomarker discovery, clinical proteomics includes the identification of new therapeutic targets, drugs and vaccines for better therapeutic outcomes and successful disease prevention. In addition to clearly defining the clinical problem, it is vital to decide whether the application of proteomic analyses will improve the current standard approaches for diagnostics and clinical care. Such considerations require specialists with clinical knowledge and, consequently, involvement of a clinician from the beginning of the study. The use of cell lines or biological samples from a clinical centre does not automatically justify labelling the proteome analysis of these samples "clinical proteomics".

The involvement of clinician(s) should enhance the precise definition of the clinical syndrome or condition of interest. Furthermore, very accurate clinical characterisation of patients and controls (high fidelity phenotyping) is mandatory to assure the highest quality clinical data – clinical samples are only as useful as the quality of their associated clinical data. It is often not justified to define potential "disease-specific biomarkers" by comparing the proteome data from a disease group to data obtained only from normal healthy individuals. To assess the specificity of the results, patients with other diseases/disorders that may have clinical, biochemical and metabolic profiles mimicking those of the disease of interest must be included. The appropriate diagnostic specimen (*e.g.* urine, blood or tissue) for the particular clinical problem should be selected and appropriate and reproducible collection procedure(s) tested. If existing sample banks are to be used, measures to ensure their probity must be developed. The discovery phase should often be seen separately from the clinical validation phase, and the types of samples required may differ for these two phases.

For any clinical study, rules and prerequisites are necessary to achieve technical and biological reproducibility, comparability and statistical robustness of the data. The scientists involved should be experienced in translational research studies, which often encompass a greater number of dependent experimental variables and more heterogeneous sample sets than more basic research. Furthermore, although the development of sample preparation protocols, data modelling algorithms, *etc*., would not be regarded as clinical proteomics studies in their own right (according to our definition proposed above), such aspects are a critical and integral part of the overall discipline.

Clinical proteomics should follow the principles and rules of clinical trials/studies

Clinical studies, and their relevance, are graded according to international guidelines (*e.g.* [6]). Several well-defined clinical study designs exist with distinct study (outcome) purposes and goals. The clinical relevance of findings from cross-sectional epidemiological studies may markedly differ from those from prospective follow-up studies, even if similar or identical "biomarkers" were employed. In other words, not every disease "biomarker" is also an outcome parameter for patients with that specific disease. As a consequence, results of epidemiological studies should undergo thorough verification in prospective controlled (intervention) trials. These trials can be graded with respect to the benefit of a therapeutic intervention, and in analogy, also for the benefit of an outcome "biomarker". For the execution of any clinical intervention trial, adherence to the widely accepted standard of "Good clinical practice"(GCP) is mandatory (for additional information see European Union Directive 2001/20/EC), and the employment of proteomics in such trials should follow these and the "Good clinical laboratory practice" (GCLP) standards [7]. GCP/GCLP compliance (and consequently establishment of standard operating protocols (SOPs), quality control (QC) protocols, traceable data management, etc.) demands more time and resources, but in return the data from clinical trials conducted in accordance with GCP/GCLP are more reliable due to extensive quality control. The obligatory input by a qualified statistician from the study initiation onwards will enhance the quality of the study design and data analysis. In the past and sometimes also nowadays, poor monitoring of data, inadequate statistical design and analysis, and dubious consent and ethical approval procedures have seriously flawed the viability of data in clinical research. As an example, although sensitivity and specificity are often quoted, an important tool in the analysis of diagnostic markers with several advantages is the receiver operating characteristic curve [8, 9] and yet this is absent from most clinical proteomic studies to date. Following or emulating GCP/GCLP standards in clinical proteomics will reduce or eliminate many of the current problems.

Finally, when proteomics is to be used in "biomarker outcome" studies, it must be tested against the current gold standard methodology (*e.g.* tissue biopsies in some clinical conditions). Only after such direct comparison can the usefulness of proteomics in clinical medicine be assessed – not only in terms of biomarker sensitivity and specificity, but also with respect to the cost effectiveness of the method and increasing the comfort of future patients. In the setting of the limited health care budgets, cost may be the most important obstacle for the widespread use of a specific proteomic analysis in clinical practice. Thus, proteomics will probably serve a dual role: first, as a primary clinical laboratory assay system to replace diagnostic procedures that are currently expensive and bear risk for the patient, such as many invasive methods, and second, as a biomarker discovery tool with the clinical assays being implemented on cheaper platforms, such as ELISA-type systems.

Design and successful completion of a clinical proteomics study requires the interaction of clinicians, statisticians/ bioinformaticians and biologists/clinical biochemists

Clinical proteomics is an interdisciplinary field and requires involvement of clinicians, statisticians/bioinformaticians, epidemiologists, clinical and analytical chemists and biologists/biochemists from the beginning, with the different responsibilities clearly stated in any subsequent report. It is naive to believe that a basic scientist or statistician will intuitively appreciate and define a clinical problem including sampling times, frequencies, *etc*. Likewise, it is unlikely that a clinician will always understand technically demanding, state-of-the-art proteomic analyses. As evident by the lack of reproducibility and generalisability of many proteome stud-

ies, inadequate bioinformatic and biostatistical measures have frequently been used, affecting aspects from study design to data analysis and interpretation.

Consideration of pre-analytical issues and assessment of reproducibility and analytical performance are essential to allow interpretation and comparison of clinical proteomics studies

Past experience has demonstrated that accounting for the large number of biological variables affecting human proteomes (*e.g.* different diseases, age, gender, lifestyle, circadian rhythms, food intake, exercise) requires consistent robust collection and handling protocols and the analysis of a prohibitively large number of samples. Making datasets from different groups and studies publicly available in data repositories (see below), thereby enabling the pooling of results from many studies and (cross-) validation of the findings and assessment in defining or narrowing down biomarkers (especially those present in many diseases) may address this problem. However, this approach will not substitute for a carefully defined study design, consideration of potential confounding factors and subsequent validation studies.

The issue of "bias" threatens the validity of biomarker studies and, hence, clinical proteomics [10]; "chance" and "generalisability" are two other major underlying issues. The avoidance of bias is not trivial and must be addressed throughout the study, from design to analysis and interpretation. Published reports must include sufficient details to allow the reader to assess the likelihood of bias influencing the results. Although we know little about the effects of normal physiological variables on proteomic studies, such variables between different groups should be minimised, for example by employing parametric matching between case and controls. Controls can be internal (samples taken at baseline in an individual) and external (matched from the population) and are often the hardest samples to obtain and clinically define. From the perspective of differential diagnosis, an additional and potentially more appropriate control group may be one consisting of patients with a disease that affects the same organ rather than healthy controls. Pre-analytical variables are often overlooked and include factors such as sample processing, handling and storage (*e.g.* samples being stored at different temperatures or for different periods of time), all of which may markedly influence the results obtained.

Underpinning the clinical qualification of any marker is the need for adequate qualification/validation of the analytical approach used at the various stages. Reproducibility is essential and, to enable evaluation and use of the data by others, values for analytical performance (accuracy, deviation, resolution, *etc.*) must be provided. The entire process (sampling, sample preparation and analytical platforms as well as electronic data evaluation) requires standardisation, quality control criteria and protocols, and validation. Currently, such protocols are absent in studies from many laboratories but are essential for meaningful clinical proteomics research. It is inappropriate to define quality standards now for each of the existing different analytical platforms, but certain basic considerations may help to define these individually for each laboratory. Issues that should be addressed are:

- Resolution of separation
- Minimal number of proteins/variables required to be detected
- Reproducibility of the analysis (*e.g.* based on 100 defined signals) when:
  - using the same sample
  - using the same sample, but different preparation
  - using different samples obtained in a limited time frame from the same individual
  - examining inter- and intraindividual variation
- Resolution of mass spectrometer
- Mass accuracy of mass spectrometer
- Operational qualification/performance verification of all used instrumentation
- Recovery of the entire process (based on, *e.g.* samples spiked with defined standards)
- Assessment of linearity (*e.g.* via serial dilution of sample) of the entire process
- Peptide sequencing validation protocols
- Detection limit of the complete process

One aspect that is often overlooked in the design of studies is the number of samples *per* group needed to detect a change with a certain degree of confidence. A statistician must be involved in defining sample numbers with stated powers of detecting a change of a specific magnitude. This feature (and the resulting power calculations) will depend greatly on the analytical performance of the particular technology and laboratory, again underlining the importance of defining the above-listed parameters before the start of any study. In some cases the discovery approach may involve a comprehensive comparison of a statistically weak number of samples. In this circumstance, a second robust screening analytical approach, perhaps involving immunoassays, will need to impart the appropriate higher level of statistical confidence to any biomarker candidates.

Estimation of the accuracy of the data must be included for adequate comparison and subsequent evaluation of the data

Clinical proteomics can be considered a sophisticated comparative analysis. When using arrays, the identity of a certain analyte is derived from its position on the array. The probability that a signal at a given position is due to a specific compound is defined by the intensity of the signal above a certain threshold value. When comparing MS data of sepa-

rated/fractionated proteomes, the identity of an analyte is defined by its migration during separation and by its mass. Both variables are defined with allowable variation. An acceptable deviation for identical analytes to be detected in different samples must be clearly defined. This can be accomplished by quality control that will include analysis of (i) intra-assay variation, represented by variation between replicates of the same sample; (ii) inter-assay variation, represented by reproducibility of results obtained on different occasions; and (iii) consistency of results obtained from different dilutions of the sample and how different samples behave in this respect (the so-called "parallelism" problem). Inevitably, comparison of proteome data will always be prone to error, but the extent needs to be determined. Depending on the approach, any methods necessary for data normalisation and processing should be specified.

Similar considerations apply for the quantification of peptides or proteins. For gels, the choice of the protein stain is important, and is based on dynamic range *vs.* sensitivity. The newer fluorescent stains have large linear dynamic ranges and similar sensitivity compared to the older Coomassie blue and silver stains [11]. There are also good MS methods for relative quantification based on ion counting [12], but care in instrument and technical consistency and acceptable mass accuracy is essential. Absolute quantification is desirable but usually requires prior identification of the biomarker sequence and/or chemical derivatisation, which consequently may become restrictive [13]. Relative quantification of biomarker abundances with reference to constant peaks is normally sufficient, especially when considering biological variation. No matter which quantification approach is used, the method and quality must be reported.

An ideal single biomarker may not exist for each disease

Many studies to date have indicated that a single protein, by itself, is generally unlikely to exist that clearly defines a specific disease (stage) distinguishing it from all other diseases or clinical conditions (for a recent review see [14]). Although there are some good examples of single markers, such as the troponins that often provide a "yes/no" diagnosis, having several markers may allow a more definite diagnosis, better disease stratification and additional clinical value. A panel of multiple potential disease-specific biomarkers is conceivable using proteomics because of the "holistic" nature of the proteomic response to disease. The hope is that by combining these biomarkers to a specific model, panel, or pattern, a certain disease can be defined with higher precision. This "molecular signature" may better define the clinical status of the individual. Such an approach requires statistical analysis that should demonstrate that a multipartite biomarker has a better predictive value than the usual routine individual markers, even when combined in the same model (*e.g.* Framingham formula [15]).

For any multiplex biomarker profiling studies, it is imperative to avoid the information on individual proteins disappearing in a "blur" of ill-defined signals. This "molecular signature" must be based on clearly defined individual proteins/peptides identified by a well-described algorithm, whether arising from precise MS analysis, gel pattern or a multiplex antibody array. An algorithm that uses all potential biomarkers in a molecular signature has yet to be found and often several different biomarker patterns can be defined. As a consequence, the biomarker pattern that is initially defined and validated in a blinded study is generally based on only a fraction of all potential biomarkers available. In future studies, some of the selected biomarkers will be found to be of lesser value, whereas others that were not previously utilised, may subsequently gain in value as studies become bigger or further supporting information becomes available. Consequently, while the analytical data are not subject to change, the biomarker pattern description will most likely change. This result does not invalidate the initial findings or their clinical usefulness, but represents an improvement. It is therefore of utmost importance that a study reports all proteins and peptides analysed rather than just the defined potential biomarkers (see also below) to allow continuing mining of these data.

Ideally, all peptide/protein biomarkers should be sequenced

Although not essential for the establishment of valid signature patterns if reliable methods for definition and detection are available (*e.g.* accurate mass and migration time), it is important that the biomarkers be identified. This is necessary from the aspect of increasing our biological knowledge about disease processes and also in terms of subsequent measurement using other technologies [16, 17]. Currently, the majority of commercial diagnostic assays are immuno-capture based, and it is very likely that any translation of the biomarkers will involve a similar format, whether the readout involves classical ELISA, multiplexed immunoassays or immuno-MS. We want to emphasise that the analysis of single biomarkers with immunological technologies requires probes specific, not merely for the native protein from which the biomarker is derived, but for the distinct biomarker that has a defined C and N terminus, as well as (frequently) PTMs. Ignoring these features may lead to false-positive results, and hence must be avoided.

Identification of proteins can be difficult, particularly for low-abundance proteins. Many "potential markers" identified in proteomic experiments have been from amongst the most abundant proteins (*e.g.* [18, 19]) and whether these will prove to be robust and/or specific still needs to be determined. A strict requirement for sequence will favour reports on only these abundant proteins and therefore appears not advisable. If the biomarker identifi-

cation workflow entails separation of intact proteins, enzymatic digestions and MS analysis of the digestion products, then standard methods for MS/MS sequencing can be used. However, these methods generally do not account for PTM, consequently further characterisation is required. Such demand is of the outmost importance, since the biomarker may be defined on the basis of C- or N-terminal truncation or a PTM, *e.g.* a specific glycoform or truncated form. In the absence of a precise definition of a biomarker in terms of sequence and PTM, a clear definition of their physicochemical properties that allows their detection in other samples (*e.g.* by affinity, p*I*, migration characteristics, mass, *etc.*) is essential. This approach stipulates that the separation process and subsequent MS analysis must be of sufficient quality to reproducibly assign relative quantities, migration in the separation dimension, and mass, all with reasonably high accuracy and resolution.

Many of the high-throughput methods directly detect native peptides and proteins present in tissues or body fluids [20, 21]. Because the termini of these naturally occurring polypeptides have not been generated by defined enzymatic cleavage and they frequently harbor PTM, direct identification of the polypeptide is challenging and often without success (*e.g.* [22]). New technologies using electron-based dissociation techniques for MS/MS have shown great promise, but are still relatively undeveloped [23]. Furthermore, some PTMs may be disease specific, and can themselves serve as biomarkers (*e.g.* advanced glycation endproducts in diabetes, [24]). In combination, these issues are a large source for errors and ambiguities in sequence assignments, and sequencing biomarkers remains a formidable, but promising area of research. Of note, it is acceptable for the clinician to use a proteomic polypeptide profile consisting of yet-unsequenced peptides for diagnosis of disease, to predict the risk of disease development and progression and/or the efficacy of treatment, if it has proven its value in blinded multi-centre and repeated studies. For this purpose the descriptive knowledge is sufficient.

Assessment of the value of potential biomarkers using blinded datasets

Proteome analysis generates multiparametric datasets, where the number of dimension/variables (different proteins analysed) usually greatly exceeds the number of samples [17]. Consequently, differences between the datasets (potential biomarkers) that enable the discrimination between any arbitrary combinations of datasets can easily be defined. This sample heterogeneity makes it impossible to thoroughly validate any biomarker or combination of biomarkers based on only the training set (initial group of samples used to identify biomarkers) even when using cross-validation. As a consequence, each clinical proteomic study must include, as one of the last and mandatory steps, the validation of the findings using an appropriate set of blinded samples analysed independently at a later time, as illustrated in several recent studies [25–27]. Only then will the data be useful for the assessment of any potential clinical benefit.

The value of clinical proteomics lies in its application

The essential and defining feature of a clinical proteomics study is to obtain robust results that lead to improvement of the current clinical situation. Such improvements may be increased sensitivity and/or specificity in diagnosis, less invasive and/or risk-associated procedures for diagnosis or therapy evaluation, or reduced costs, to name just a few. The results must be clinically relevant and translatable into clinical practice. A clear understanding of the application of the proteomic test in a clinical setting is needed, in terms of which patients may benefit and what impact the test may have on the outcome of the disease. Such demand requires a clear definition and validation of the assay (technological platform in combination with the biomarkers) to be used for clinical application. Certain platforms (*e.g.* CE-MS, MALDI-TOF) may be utilised for discovery, validation, and potentially subsequent clinical application, omitting the need for subsequent translation to another assay system. Others, such as protein separation by 2-DE or protein and peptide separation by multidimensional LC-MS are primarily research and discovery tools and currently not applicable for clinical diagnosis, because they are low-throughput and time-consuming technologies. While these approaches are very powerful in the discovery process, the results must be translated and validated on an appropriate "clinical platform" (*e.g.* ELISA, multiplex immunoassay, *etc.*) as a prerequisite for publication as a clinical proteomics study. Further, the establishment of the "clinical platform" is an essential part of clinical proteomics and should be addressed in any proposal/study plan.

Public and open access to data is required to bring clinical proteomics to its full potential

Past experience has shown that multivariate analyses (as proteomics generally is) require an enormous wealth of data. Ideally, tens of thousands of datasets must be obtained and evaluated. Such a task requires data sharing in a standardised exchange format among different groups. This is probably best accomplished by a proteomics database, which must adhere to strict rules to ensure desired clarity and quality [28]. The common format should include all proteome data (*e.g.* peak lists), linked when possible with other characteristics obtained from fractionation (*e.g.* migration or retention time at specific conditions,

p*I* or molecular mass) together with the study protocol, sampling procedure and sample preparation, calibration, matching and quality control criteria, as well as clinical data. Such a database does not currently exist in the public domain. Therefore, we recommend that such a database be established, *e.g.* by building on the existing PRIDE (Proteomics Identification) database at EBI. In a manner similar to the obligatory registration of clinical trials at international trial registries (*e.g. clinicaltrials.gov* operated by the U.S. National Institutes of Health), deposition of proteomic data should become mandatory for publication and as part of the funding requirement for academics. Such a degree of data sharing will add enormous value to the basic essence of a clinical proteomics study because these data could serve as additional controls or validation sets for unrelated studies. However, although highly desirable, great thought should be given to the implementation of such data-sharing, as the real possibility of abuse of the data for non-intended purposes does exist.

Table 1. Recommended steps for clinical proteomics

1) Define a clear clinical question and how the outcome of the study would improve the diagnosis and/or treatment of the disease
2) Define the patient and control populations, clinical data to be collected, as well as protocols for sampling and sample preparation
3) Define the type of samples needed for the discovery and validation phases
4) Define and validate the analytical platforms for discovery (those for validation may well differ)
5) Obtain IRB approval and written informed consent from the participant
6) Perform a pilot study on a validated discovery platform
7) Statistically evaluate data from the pilot study to calculate the number of cases and controls for the training set
8) Perform study of the training set on the validated platform based on the calculated number of cases and controls
9) Evaluate findings from the training set on blinded samples
10) Deposit datasets in a public database
11) Using these results, transfer the assay to the application platform and test using a training (if applicable) and subsequently a blinded set
12) Apply towards clinical use to show whether the findings improve the current clinical situation

Steps 1–4 could serve as a preliminary study that may be the mandatory basis for funding an application. Steps 5–8 can be seen as the actual clinical proteomics study, which, even if no positive result was obtained, should be published and the data deposited in an openly accessible database. Step 11 is obligatory if the validated discovery platform is identical to the validated application platform. Omission of any of these steps could potentially invalidate the study and hence should be avoided.

Data reporting: what is publishable in the field of clinical proteomics?

Most of us face the pressure to publish in high-impact journals to scientifically survive. This pressure clearly generates the problem of "over-interpretation" of data, undesirable in general, and especially deleterious in clinical proteomics, as past experience has shown. It would be helpful if a well-performed study could be published even if no significant and valid biomarker(s) were found. The valid data would be available for comparison, and unrealistic high hopes would be tempered. Along these lines, the aims in any clinical proteomics study must not be overly ambitious (such as requiring that all potential biomarkers be sequenced, classification exceeding accuracy of 90%, *etc.*), and such currently unrealistic demands should neither be required by expert reviewers nor by editors as prerequisites for publication. However, standards, study design, data analysis/interpretation, statistical evaluation, *etc.* (see Tables 1 and 2), must be defined in any publication. The negative effects of inappropriate claims about biomarkers adversely impact not only on the scientific community but can also have marked effects on patients' expectations.

Where does clinical proteomics stand today?

Several recently published studies indicate that clinical proteomics has grown beyond infancy and the results can be utilised to tackle current clinical problems when studies are carefully designed with appropriate controls in place at both the pre-analytical and analytical stages (*e.g.* [17, 25, 26, 29]). While clinical proteomics will improve further, we must bear in mind that the goal is to benefit the patients as soon as possible, *i.e.* not to wait for a perfect system with "infinite" sensitivity and accuracy. However, we have a duty to ensure that expectations of patients and clinicians are not raised erroneously high, and so key evidence-based progression is essential. As soon as sufficient evidence supports a biomarker as having sufficient clinical benefit in initial validation studies, the next stage of the development pipeline should be addressed, *i.e.* its clinical use in larger clinical studies.

Concluding remarks

In the past, most studies have not complied with many of the recommendations discussed here, frequently due to high enthusiasm from a diverse audience that may have lacked direct knowledge and experience. However, recent studies indicate that potentially successful marker discovery conform to or approach the suggested guidelines included here. Our proposed guidelines are by no means complete and will be subject to change as technology and the field evolves,

Table 2. Suggested essential reporting requirements for publication of a clinical proteomics study

Aspect	Information required
Study aims	Clear definition of the overall and specific clinical aims of the study and statement of the study design, *e.g.* cross-sectional, longitudinal, *etc.*
Study groups	Description of the sizes and composition of the groups to include basic demographic data, such as age and gender but also relevant associated clinical data summaries. As an example, for a certain cancer study, this may include details of the histological subtypes, stages and grades and any treatment. Such information should be provided for the initial analysis groups and also for subsequent validation samples.
Samples	Details of the types of sample, the timing of collection relative to the disease process (*e.g.* pre-operative or pre-treatment) and how they were processed (*e.g.* blood/sample tube manufacturer, elapsed time between sample collection and processing, centrifugation/processing details, temperature of processing and storage and length of storage) and whether any particular additional measures were taken, such as fasting, *etc.* This information should be provided for the samples obtained from each study group.
Sample size and design of analysis protocols	Explanation of how the numbers in each group were selected in terms of statistical power calculations used, previous findings, *etc.* Detailed information should be provided about replicate numbers, whether investigators were blind to sample groups and whether randomisation was used for the analysis of samples.
Analysis	Full details of the analytical approach including any sample pre-fractionation. Exact details will be approach-dependent but should allow sufficient details for the method to be reproduced. Information provided should also allow assessment of the reproducibility and accuracy of the approach. As examples, %CVs between gels or LC elution profiles should be provided and for MS-based profiling, CVs for all data including the mass and intensity for peaks, as well as additional predefined acceptance criteria (*e.g.* lower limit of quantification) should be provided with full details of calibration protocols.
Quality control	All quality control methods should be described.
Protein identification	Any MS protein identification should conform to the recent "Paris guidelines" for the presentation of such data [30].
Data processing, data analysis and data reporting	All bioinformatic and biostatistical methods used for quality control, data processing or data analysis should be described in detail or appropriately referenced. The data reporting should in general follow the MIAPE guidelines of the HUPO Proteomics Standards Initiative.
Validation	For studies where the identity of the biomarkers is known, results should be validated using an independent sample set, either using the original technique or an alternative assay such as an immunobased assay or array. For studies using computer-based models of profiles in which the identity of the biomarkers is unknown, it is essential that the model be validated with an independent test set of samples analysed during a completely separate time period.

but they are an initial starting place that should stimulate discussion and refinement. While it is clear that proteomics can make valuable contributions to clinical questions today, the current challenge and long-term goal is to explore the most effective ways in which proteomics can be integrated with current clinical laboratory medicine to maximise their synergism for the benefit of the patient. The impact of clinical proteomics will depend on the choice of samples, their technical quality, transport, storage, and analysis, as well as on the clear definition of clinical problems and scientifically sound questions in the discovery and clinical validation phases. As evident from the first successful studies, if basic standards are followed, the transfer of recent advances in proteomic technologies to the clinical arena has the real potential to provide major improvements for the diagnosis and treatment of various diseases.

References

[1] Vitzthum, F., Behrens, F., Anderson, N. L., Shaw, J. H., *J. Proteome Res.* 2005, *4*, 1086–1097.

[2] Rifai, N., Gillette, M. A., Carr, S. A., *Nat. Biotechnol.* 2006, *24*, 971–983.

[3] Hernandez, J., Thompson, I. M., *Cancer* 2004, *101*, 894–904.

[4] Pepe, M. S., Etzioni, R., Feng, Z., Potter, J. D. *et al.*, *J. Natl. Cancer Inst.* 2001, *93*, 1054–1061.

[5] Baker, S. G., Kramer, B. S., McIntosh, M., Patterson, B. H. *et al.*, *Clin. Trials* 2006, *3*, 43–56.

[6] American Society of Clinical Oncology, *J. Clin. Oncol.* 1996, *14*, 671–679.

[7] Stiles, T. G. V. and Mawby, N., *Good Clinical Laboratory Practice (GCLP)*, BARQA, Ipswich

[8] Obuchowski, N. A., Lieber, M. L., Wians, F. H., *Clin. Chem.* 2004, *50*, 1118–1125.

[9] Baker, S. G., *J. Natl. Cancer Inst.* 2003, *95*, 511–515.

[10] Ransohoff, D. F., *Nat. Rev. Cancer* 2005, *5*, 142–149.

[11] Shaw, J., Rowlinson, R., Nickson, J., Stone, T. *et al.*, *Proteomics* 2003, *3*, 1181–1195.

[12] Wang, W., Zhou, H., Lin, H., Roy, S. *et al.*, *Anal. Chem.* 2003, *75*, 4818–4826.

[13] Ong, S. E., Mann, M., *Nat. Chem. Biol.* 2005, *1*, 252–262.

[14] Vasan, R. S., *Circulation* 2006, *113*, 2335–2362.

[15] Anderson, K. M., Wilson, P. W., Odell, P. M., Kannel, W. B., *Circulation* 1991, *83*, 356–362.

[16] Malik, G., Ward, M. D., Gupta, S. K., Trosset, M. W. *et al.*, *Clin. Cancer Res.* 2005, *11*, 1073–1085.

[17] Theodorescu, D., Wittke, S., Ross, M. M., Walden, M. *et al.*, *Lancet Oncol* 2006, *7*, 230–240.

[18] Schaub, S., Wilkins, J. A., Antonovici, M., Krokhin, O. *et al.*, *Am. J. Transplant.* 2005, *5*, 729–738.

[19] Candiano, G., Musante, L., Bruschi, M., Petretto, A. *et al.*, *J. Am. Soc. Nephrol.* 2006, *17*, 3139–3148.

[20] Schiffer, E., Mischak, H., Novak, J., *Proteomics* 2006, *6*, 5615–5627.

[21] Semmes, O. J., Feng, Z., Adam, B. L., Banez, L. L. *et al.*, *Clin. Chem.* 2005, *51*, 102–112.

[22] Zurbig, P., Renfrow, M. B., Schiffer, E., Novak, J. *et al.*, *Electrophoresis* 2006, *27*, 2111–2125.

[23] Coon, J. J., Ueberheide, B., Syka, J. E., Dryhurst, D. D. *et al.*, *Proc. Natl. Acad. Sci. USA* 2005, *102*, 9463–9468.

[24] Lapolla, A., Tubaro, M., Fedele, D., Reitano, R. *et al.*, *Rapid Commun. Mass Spectrom.* 2005, *19*, 162–168.

[25] Semmes, O. J., Cazares, L. H., Ward, M. D., Qi, L. *et al.*, *Leukemia* 2005, *19*, 1229–1238.

[26] Decramer, S., Wittke, S., Mischak, H., Zurbig, P. *et al.*, *Nat. Med.* 2006, *12*, 398–400.

[27] Wittke, S., Haubitz, M., Walden, M., Rohde, F. *et al.*, *Am. J. Transplant.* 2005, *5*, 2479–2488.

[28] Taylor, C. F., Hermjakob, H., Julian, R. K. Jr., Garavelli, J. S. *et al.*, *OMICS* 2006, *10*, 145–151.

[29] Roessler, M., Rollinger, W., Mantovani-Endl, L., Hagmann, M. L. *et al.*, *Mol. Cell. Proteomics* 2006, *5*, 2092–2101.

[30] Bradshaw, R. A., Burlingame, A. L., Carr, S., Aebersold, R., *Mol. Cell. Proteomics* 2006, *5*, 787–788.

Addendum

[2] European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, UK; [3] Cancer Research UK Clinical Centre, St James's University Hospital, Leeds, UK; [4] Department of Health Evaluation Sciences, University of Virginia, Charlottesville, VA, USA; [5] Departments of Chemistry and Biomolecular Chemistry, University of Wisconsin-Madison, Madison, WI, USA; [6] BHF Glasgow Cardiovascular Research Centre, University of Glasgow, Glasgow, UK; [7] Hannover Medical School, Department of Pediatrics, Hannover, Germany; [8] Hannover Medical School, Department of Nephrology, Hannover, Germany; [9] Department of Computing Science, University of Glasgow, Glasgow, UK; [10] Charité (CBF), Medizinische Klinik IV, Berlin, Germany; [11] Biomedical Proteomics Research Group, Department of Structural Biology and Bioinformatics, Faculty of Medicine, University of Geneva, Geneva, Switzerland; [12] Charite-Universitatsmedizin Berlin, Berlin, Germany; [13] Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, USA; [14] Beatson Institute for Cancer Research, Glasgow, UK; [15] INSERM ERI-12, Amiens, France and Amiens University Hospital, UPJV, Amiens, France; [16] University of Aalen, Aalen, Germany; [17] Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA; [18] Baker Heart Research Institute, Melbourne, Australia; [19] Steno Diabetes Center, Gentofte, Denmark; [20] Inserm, U388, Toulouse, France; [21] Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, Norfolk, VA, USA; [22] Department of Urology and Mellon Prostate Cancer Institute, University of Virginia, Charlottesville, VA, USA; [23] Faculty of Medicine at Siriraj Hospital, Mahidol University, Bangkok, Thailand; [24] Hannover Medical School, Department of Hematology, Hemostasis and Oncology, Hannover, Germany; [25] Department of Medicine, Johns Hopkins University, Baltimore, MD, USA; [26] Department of Structural Pathology, Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

